IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 3672
Astsaturov, et al.	Art Unit: 1632
Appl. No. 10/690,199	Examiner: Wu C.W. Shen
Filed: 10/21/2003	
For: Vaccines Using High-Dose Cytokines	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.131

I, Neil Berinstein, a citizen of Canada, declare as follows:

1. I am an inventor on the present application ("our application"), together with Igor Astasaturov, Teresa Petrella, Mark DeBenedette, and David E. Spaner.

2. I have been involved in cancer research as Head of the cancer vaccine program at Sanofi Pasteur, and as a clinician-scientist and Professor of Medicine and Immunology at the University of Toronto and Sunnybrook Hospital since 1988. At the time the invention described in our patent application was made, I was an employee of Sanofi Pasteur.

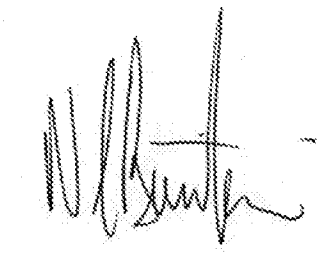
3. I have reviewed the pending claims of our application. Our claims relate to a method for administering to a mammal a composition comprising a nucleic acid encoding melanoma-associated tumor antigen, and subsequently administering to that mammal one or more high doses of interferon alpha 2b (IFN-α2b). This corresponds with what is described in Examples 1 and 2 of our application.

4. Our U.S. provisional patent application was filed on October 22, 2002. However, we conceived of and began to reduce to practice our claimed invention prior to that date, as described in point 5.

5. I contacted Dr. Patrick J. Halloran, our patent attorney at Sanofi Pasteur, at least as early as July 9, 2002 to describe our invention and discuss the possibility of preparing a patent application. At that time, I submitted a draft manuscript (attached as **Appendix A**) describing our invention to Dr. Halloran.

6. During the entire time between at least July 9, 2002 and the filing date of our patent application, I believe we acted with diligence in developing our invention.

7. All statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application and any patent issuing thereon.



September 29, 2010

Neil Berinstein, M.D. — Date

APPENDIX A

HIGH DOSE INTERFERON-α2B AMPLIFIES VACCINE INDUCED ANTI-TUMOR T CELL RESPONSES: IMPLICATIONS FOR CANCER VACCINE STRATEGIES

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***I.A. and T.P. share first authorship.**

Running title: Increased potency of anti-tumor responses induced by Interferon-α after vaccination

These studies were supported by a grant from the Canadian Institutes of Health Research (CIHR) to DS. DS is a physician-scientist supported by Cancer Care Ontario.

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Abstract: Cancer vaccines are potentially of therapeutic benefit to patients who cannot be cured with conventional chemotherapy. A problem with current vaccines formulated from defined tumor antigens, that are also self-antigens, is that the relatively weak responses of the activated T cells are not maintained for long enough to give a therapeutically meaningful result. We administered high dose Interferon-α2a (IFN-α) (HDI) ($20x10^6$ U/m^2) for one month to 7 high-risk melanoma patients who had previously been injected with a vaccine based on immunodominant HLA-A*0201 binding epitopes of the melanoma antigen, gp100. HDI recalled gp100-reactive T cells in the 4 patients who had previously (but transiently) responded to the vaccines but not in the remaining patients in whom a previous response was not detectable. In two patients, HDI was associated with disappearance of metastatic lesions, even when IFN-α had been used for therapeutic purposes before vaccination. Moreover, only the population of gp100-reactive T cells recalled by HDI was able to kill gp100-expressing tumor targets. These results suggest that HDI recalls previously activated self- and tumor-reactive T cells that are able to become potent killers. The results help to explain the association of infections (that cause the release of endogenous IFN-α) with the development of autoimmune diseases and also suggest a strategy to maintain anti-tumor responses initiated by cancer vaccines.

Introduction: Cancers, such as melanoma, that are incurable with conventional chemotherapy[1] may be susceptible to treatment with vaccines that enhance the activity of tumor-reactive T cells.[2] In the last few years, a number of tumor antigens have been identified and used to make specific cancer vaccines. For melanoma, these antigens include members of the MAGE family, tyrosinase, gp100, melanA/Mart-1, and Trp-2.[3-5]

Despite the identification of these defined tumor targets, therapeutic results with cancer vaccines have been largely disappointing. While it has been relatively easy to transiently activate tumor-reactive T cells with vaccines,[6] these responses are often not maintained for sufficient time to provide significant therapeutic benefits.[7] Reasons for this transient activation include: i. tumor-reactive T cells are often only weakly reactive to tumor antigens that are self-antigens. ii. T cells exposed to these antigens during tumor progression may have become anergic. iii. immuno-regulatory controls that prevent sustained auto-immune responses may also inhibit anti-tumor responses.[7,8]

We have recently examined the effects of vaccines, containing the tumor antigen, gp100, on melanoma patients at high risk of developing metastatic disease because of the depth of their primary lesion or because of lymph node or other involvement.[9] In agreement with other studies, we found that the resulting anti-gp100 T cell responses were often transient.[7] In order to provide additional therapeutic benefits to these patients, several were administered high dose Interferon-α2b (HDI) for one month.

Interferon-α2b (administered intravenously (IV) at high doses ($20x10^6 U/m^2$) 20 times over 4 weeks) and then subcutaneously (SC) at low doses ($10x10^6 U/m^2$) 3 times per week for 48 weeks) has been shown to significantly prolong relapse-free survival and overall survival compared to observation alone in high-risk melanoma patients (Stage IIB

and III).[10,11] Since trials that used only SC injections failed to show such a survival benefit, it is hypothesized that the high dose component provides the most important therapeutic element of IFN-α.[10] This hypothesis led to the design of a phase I clinical trial to determine if anti-tumor responses induced by the vaccines could be improved by high dose IFN-α (HDI).

We found that if patients had previously demonstrated an immune response to the vaccine, it could be recalled by HDI. Importantly, the quality of this response was different. In contrast to the T cells activated by the vaccines, alone, tumor-reactive T cells recalled by HDI were able to kill tumor antigen-bearing targets *in vitro* and were associated with more profound therapeutic effects *in vivo*. These results lend insight into the mechanism of action of IFN-α in cancer therapy and have implications for the pathogenesis of auto-immune diseases, in general. They also suggest that HDI after vaccination with tumor antigens is an effective immunotherapeutic strategy.

Materials and Methods

Patients: For entry into this study, patients were required to have histologically confirmed malignant melanoma at high risk of developing metastases (pT3 or higher, any N, any M by AJCC staging),[11] have the HLA-A*0201 haplotype, be older than 18 with an ECOG performance status of 0 or 1, and give informed, written consent according to national and institutional guidelines before treatment. All patients in this trial had completed a vaccination protocol as part of a phase I trial sponsored by Aventis-Pasteur. The vaccine protocol involved injections of the ALVAC (2)-gp100M recombinant virus (an investigational product of Aventis-Pasteur made from a second generation Canarypox virus expressing a full length gp100 gene encoding two epitopes modified for enhanced HLA class I binding) along with the two modified peptide epitopes.[12,13] Full details of this vaccine trial, which is still ongoing, will be published elsewhere.

Treatment with HDI: IFNα2b (Schering Canada, Pointe-Claire, Quebec) was administered using the dose and schedule previously tested.[14] HDI consisted of 20 MU/m^2/d IV 5 days/week x 4 weeks. The IFNα2b dose was held and then restarted at a 33% dose reduction if severe toxicity (grade 3 or 4, defined by the common toxicity criteria established by the National Cancer Institute Cancer Treatment Evaluation Program[15]) was observed. A second decrease of 33% of the original dosage was made in some patients for recurrent severe toxicity.

Study design: After being discharged from the Aventis-sponsored vaccine trial, but still considered to be at high-risk for developing metastatic disease, patients were administered HDI after giving informed consent. Patients were monitored for toxicity weekly during the first month of study (while on HDI) and then for toxicity and disease status at monthly intervals for 3 months. Radiologic evaluation was performed at 3 months of follow-up to assess tumour response. Peripheral blood was collected for immunological monitoring at each time point in sodium heparin containing tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Hypaque 1.077 (Amersham Pharmacia, Sweden). Cells were washed twice in phosphate buffered saline (PBS) and frozen in 10% DMSO and 90% autologous heat-inactivated plasma (56°C for 30 min). Aliquoted cells were kept in liquid nitrogen until use.

Reagents: **Peptides:** Peptides (provided by Aventis Pasteur, Toronto, Canada) corresponded to the influenza (FLU) matrix protein MP, residues 58-66, (GILGFVFTL),[16] two dominant epitopes from the melanoma antigen, gp100, modified to increase binding to class I MHC, gp100:209-2M (IMDQVPFSV) and gp100:280-9V (YLEPGPVTV),[17] and the HIV p17 Gag protein derived peptide (SLYNTVATL).[18] All peptides were HLA-A*0201 restricted CTL epitopes. The gp100 peptides were dissolved in water (5 mg/ml stock solution) and the others were dissolved in DMSO (Sigma, St. Louis, MO) (10 mg/ml stock solution). **Antibodies and tetramers:** CD8-FITC antibodies were purchased from PharMingen (San Francisco, CA). BB7.2 (anti-HLA-A2)[19] hybridomas were obtained from the American Type Culture Collection (ATCC)

(Vanassa, Va). Antibodies from this hybridoma were purified, and labeled with FITC, in our laboratory. Three purified, soluble recombinant HLA-A*0201-peptide complexes bound to phycoerythrin (PE)-labeled streptavidin[20] were purchased from ProImmune Ltd. (Oxford, UK). The peptide sequences were YLEPGPVTV (Lot No. BL/0757), IMDQVPFSV (Lot No. BL/0755), and GILGFVFTL (Lot No. BL/0839). **Cell Lines.** Human lymphoblastoid T2 cells were obtained from the ATCC. Deletion of the TAP-transporter gene locus in T2 cells prevents delivery of cytoplasmic peptides into the ER.[21-23] As a result, surface HLA expression is defective but can be rescued with exogenous peptides. The large number of identical peptide-MHC complexes on peptide loaded T2 cells make them potent antigen presenting cells (APCs).

HLA-typing: HLA-A2$^+$ patients were identified using flow cytometry and BB7.2 antibodies. Molecular subtyping of HLA-A2 was performed by the HLA-laboratory at Aventis-Pasteur, using sequence-specific primer-PCR.[24]

Short term in vitro stimulation of PBMC: Cryopreserved PBMC were thawed, washed, and incubated overnight in AIM-V medium (Gibco, Burlington, Ontario) at 37°C in 5% CO_2. Cells were counted the next day in a hemocytometer and 1 ml of a cell suspension, adjusted to 2-3 x10^6 cells/ml in AIM-V plus 10% AB serum (Sigma, St. Louis, MO) (complete media, CM), was plated in single wells of a 24 well polystyrene tissue-culture grade plate (Becton Dickinson Labware, Franklin Lakes, NJ). The cultures were then either stimulated with the gp100 peptides (gp100:209-2M and gp100:280-9V, together), at a final concentration of 25 μg/ml or with MP58-66 peptides added at a final

concentration of 10 μg/ml. IL-2 (50 IU/ml) (Chiron, Emeryville, CA) was added on days 3 and 6 after peptide stimulation. At the end of the 8-9 day culture period, cells were harvested and washed before further testing.

ELISPOT assays: HA-multiscreen plates (Millipore, Bedford, MA) were coated overnight at room temperature with 75 μl of anti-IFN-γ mAb from the 1-DIK clone (MABTECH, Stockholm, Sweden) (2 μg/ml in PBS). The plates were then washed with PBS, to remove unbound antibody, and blocked with 0.5% BSA/PBS for 1 h at room temperature. PBMC were added in duplicate or triplicate wells in the presence or absence of peptide. The two modified gp100 peptides were added at a final concentration of 25 μg/ml and the FLU peptides were added at a final concentration of 10 μg/ml. Mitogenic stimulation was performed with phorbol myristic acetate (PMA) (20 ng/ml) (Sigma) and Ionomycin (1 μg/ml)(Calbiochem, San Diego, CA). IL-2 (100 IU/ml) was included in all cultures unless stimulated by mitogens. After incubation at 37°C in 5% CO_2 for 24 h, the cells were discarded, and the plates were washed extensively with 0.05% Tween/PBS. Secondary biotinylated anti-IFN-γ mAbs (clone 7-B6-1, MABTECH) were then added (75 μl/well at 1 μg/ml) and left for 2 h at room temperature, followed by extraavidin-conjugated alkaline phosphatase (Sigma) for an additional 1 h. The plates were developed using NBT/BCIP phosphatase substrate solution (Sigma) and counted using a stereomicroscope at 40x and an automated ELISPOT reader (Carl Zeiss Vision, Germany). Statistical analysis was carried out using Microsoft Excel software.

Chromium release assays for cellular cytotoxicity: T2 tumor targets in exponential growth phase were collected by centrifugation and incubated with 2 μg/ml of peptides (g209M and g280V, mixed 1:1, or FLU peptide) for 2 h at 37°C and then washed twice to remove free peptide. The cells were then resuspended in two drops of 100% fetal calf serum, and radiolabeled with 50 μl of sodium chromate (7.14 mCi/ml) (Dupont, NEN, Boston, MA) for 1 h. Effector cells, purified from the 8 day peptide stimulated cultures by density centrifugation over Ficoll-Hypaque columns, were added at varying effector: target ratios in 100 μl of CM to individual wells of a U-bottom plate. Chromium labeled targets were washed 3 times with α-MEM+1% FCS and 100 μl of target cells ($2x10^4$/ml in CM) were added to each well. The plates were centrifuged at 600 rpm for 3 min and then incubated at 37°C for 4 h. Plates were then centrifuged at 800 rpm for 5 min and 100 μl of the supernatant transferred to Fisherbrand flint glass tubes (Fisher Scientific, Pittsburgh, PA) and counted in a γ-counter (CompuGamma Model 1282, LKB, Stockholm, Sweden). Total release (TR) was measured by lysis of tumor targets with 1% acetic acid and spontaneous release (SR) was measured in the absence of effector cells. Percent cytotoxicity was determined by the ratio (cpm-SR)/(TR-SR) x 100%.

Immunofluorescence: Cell staining was performed as previously described using cells taken at the end of the *in vitro* culture period.[25]

Results:

Toxicity: As shown in Table 1, seven HLA-A*0201$^+$ patients received one month of high dose interferon (HDI) between 1.5 months and 17 months (mean=7.2±4.9 S.D.) after their last injection of a vaccine containing gp100 and its known HLA-A*0201 binding epitopes.[17,26] All 7 patients completed the course of HDI and no evidence of disease progression was noted. In fact, two patients (M166 and M335) developed marked disease reduction after HDI and their clinical course will be described in greater detail below. Patients developed typical toxicities associated with HDI including flu-like symptoms, cytopenias, and liver function test abnormalities, which lasted only during the time of HDI (Table 2). One patient (M160) developed neuro-psychiatric symptoms, requiring the institution of anti-depressants, which also cleared within a week of stopping HDI. One patient (M335) developed vitiligo around skin deposits of melanoma (described below). Dose reductions and treatment delays due to toxicity were experienced by all 7 patients (Table 2) which is somewhat higher than the 33% incidence reported for 396 patients in the E1694 Intergroup trial.[15]

Recall of vaccine-induced anti-gp100 T cell responses by HDI: The design of this study, which used HLA-A*0201$^+$ patients previously immunized with gp100 based vaccines, made it relatively easy to monitor the immunological events associated with the subsequent administration of HDI.[20] Tumor-reactive T cells could be enumerated in ELISPOT assays,[27,28] that determine the frequency of T cells that secrete IFN-γ after stimulation by the two immunogenic HLA-A*-0201 binding gp100 peptides (gp100:209-2M and gp100:g280-9V) and also by flow cytometry using tetramers of recombinant

HLA-A*0201 folded around the gp100 peptides.[29] None of the patients had evidence of circulating gp100-reactive T cells by any of these two assays before beginning the month of HDI (Fig. 1a and b, "Follow-up" dot-plots; Fig. 3b; Fig. 5; and data not shown). However, 4/7 patients had a measurable increase in the frequency of gp100-reactive T cells (arbitrarily set at $>1/10^4$ cells) at some point during the vaccination protocol (Table 1, column 7 and Fig. 1, "On Vaccine" dot-plots), although this increase was only transient (Fig. 1, "Follow-up" dot-plots; Fig. 3b; Fig. 5; and data not shown). In these patients, measurable frequencies of gp100-reactive T cells again developed by the second week of HDI (Table I column 7; Fig. 1, "IFN-α2b" dot-plots; Fig. 3b; Fig. 5). However, if patients had not achieved a measurable anti-gp100 response to vaccination, treatment with HDI did not lead to a measurable increase in gp100-reactive T cells (Table 1, column 7, patients M126, M246, and M260). As a control to ensure that the failure to demonstrate gp100-reactive T cells in these patients was not due to technical difficulties associated with the cryopreservation and culture conditions, the response to the HLA-A*0201 binding peptide, influenza (FLU) matrix protein MP, residues 58-66, (GILGFVFTL), was measured at the same time using IFN-γ ELISPOT assays and peptide-folded tetramers (Fig. 3c). It is known that 60-70% of patients have memory T cell responses to FLU from previous natural infections with this virus.[16,30] In all cases, the culture conditions were sufficient to support the development of FLU-reactive T cells (Fig. 3c and data not shown), suggesting that the absence of gp100-reactive T cells in the blood of these patients was real. Interestingly the FLU-responses did not always increase when the patients were treated with HDI compared to the baseline values (Fig. 3c and data not shown).

Association of increased gp100-reactive T cells after HDI and clinical responses in M166: One patient (M166) was a 31 year old male who initially presented with a 0.6 mm deep melanoma in his neck. Six years later, he developed a small bowel obstruction from a mesenteric metastatic melanoma deposit that was resected surgically. No other metastatic disease was evident until he was considered for the melanoma vaccine study 18 months later and found to have a mass in the gluteal region (Fig. 2a, arrow). A clinical decision was made to observe the mass during the vaccination period because of the difficult nature of the surgery required for its resection. Three months after completing active vaccination, the mass was somewhat smaller (Fig. 2b, arrow). The patient received HDI 3 months after that, the mass subsequently disappeared, and has not recurred as of 8 months later, at the time of the last follow-up visit (Fig. 2c, arrow).

As shown in Fig. 1a and Fig. 3a, M166 mounted an immune response to the gp100-based vaccines. During vaccination, gp100-reactive $CD8^+$ T cells comprised 1% of the total cells in an 8 day culture of PBMC primed with gp100:109-2M and gp100:g280-9V as measured by tetramer staining and flow cytometric analysis (Fig. 1a, "On Vaccine" dot-plot). At the end of the vaccination period, the frequency of gp100-reactive T cells fell (Fig. 3A) and disappeared by the time that HDI was instituted (Fig. 1a, "Follow-up" dot-plot; Fig. 3b). However, after one week of HDI, the frequency of IFN-γ producing gp100-reactive T cells increased to ~1/1000 (Fig. 3b) and the number of $CD8^+$ T cells that were stained by the tetramers of HLA-A*0201 and the gp100 peptides was 4.2% of the cells in the culture (Fig. 1a, "IFNα2b"dot-plot). Although the frequency of gp100-reactive T cells in the ELISPOT assay varied, it was still elevated 4 months

after completing HDI (Fig. 3b). In this patient, FLU-reactive $CD8^+$ T cell frequencies were relatively constant despite HDI and the changing gp100-reactive T cell frequencies (Fig. 3c).

Association of increased gp100-reactive T cells after HDI and clinical responses in M335: A similar result was observed in M335, a 31 year old female who had initially presented with a 0.65 mm primary lesion on her right thigh. Six years later she developed right inguinal lymph node involvement, which was resected, and she received treatment for one year with the immunomodulatory agent, levamisole.[31] Subsequently, she developed two subcutaneous metastases and was treated with HDI and 10 months of SC IFN-α2b at low doses. One year later she developed a right axillary mass which was dissected and treated with adjuvant radiation. Shortly thereafter, she had involvement of the skin and dermis of the right breast and chest wall, which was treated by mastectomy and local radiation. She was then enrolled in the melanoma vaccine trial, at which time she had developed multiple small melanotic skin metastases over the right chest but no detectable systemic disease otherwise (Fig. 4a, d). Over the 12 weeks of the schedule of vaccine injections, she developed a 4 cm mass in the scar line of the mastectomy (not shown) and adenopathy in the left axilla (Fig. 4b) and cervical region. In addition, lung nodules were found, compatible with metastases (Fig. 4e). Six weeks after the last vaccine injection, she was started on HDI. Within 2 weeks, the palpable masses in the chest wall and left axilla had disappeared, as confirmed by the CT scan taken 2 months after completing HDI (Fig. 4c). Radiologic evidence of lung metastases (Fig. 4e) also disappeared (Fig. 4f). The patient has again been maintained on SC IFNα2a and, at the

time of her last clinic visit, had no evidence of systemic metastases, except for the skin deposits. Interestingly, many of these had developed evidence of local vitiligo suggestive of auto-immune destruction of nearby normal melanocytes.[32]

Similar to M166, M335 transiently responded to vaccination, as measured by tetramers and ELISPOT assays, but this response was lost by the time that HDI was instituted (Fig. 1b, Fig. 5). However, within 2 weeks of starting HDI, and concomitant with the observed clinical response (Fig. 4), the frequency of IFN-γ producing gp100-reactive T cells increased to ~1/351 and the percentage of tetramer-staining $CD8^+$ T cells increased to ~7% of cultured PBMC by the third week of HDI. Elevated responses in these assays were maintained for at least one month after completing HDI (Fig. 1b and Fig.5).

HDI alters the quality of the anti-tumor T cell response: The frequency of gp100-reactive T cells recalled by HDI was not significantly different from the frequency that was found in response to the tumor vaccine (Table 1, columns 7 and 8; Fig. 1; Fig, 3a,b; Fig. 5). We wondered if the anti-tumor response recalled by HDI was more potent than the response that developed after vaccination to account for the therapeutic effects seen in M166 and M335. It is generally believed that TH1/TC1 responses that result in the activation of cytotoxic T cells (CTLs) able to kill tumor cells are required for optimal anti-tumor immunity.[35] Although IFN-γ production, as measured in the ELISPOT assays, is a surrogate marker for $CD8^+$ CTL function[28] we directly examined the ability of gp100-reactive T cells from M166 and M335, during vaccination or during HDI, to kill targets expressing HLA-A*0201 molecules and gp100 peptides. Since melanoma cell lines from these patients were not available, peptide-loaded T2 cells were used as targets. T2 cells

express complexes of peptides and HLA-A*0201 molecules on their cell surface only when HLA-A*0201 binding peptides are provided, because of a genetically defective TAP-transporter system.[21-23] If gp100-reactive T cells are unable to kill gp100 peptide loaded T2 cells, it seems unlikely they could kill autologous melanoma cells with a much lower surface density of gp100 peptide-HLA-A*0201 complexes.

Despite the similar frequencies of tetramer staining and IFN-γ producing gp100-reactive T cells, there were striking differences in the ability to kill gp100-peptide loaded T2 cells after HDI. Tumor-reactive T cells activated by vaccination alone were unable to kill gp100 peptide-loaded T2 cells (Fig. 6a for M166 and Fig. 6b for M335, graphs "After vaccine"). However, gp100-reactive T cells during and after HDI from both patients were potent killers of gp100 peptide-loaded T2 cells (80% lysis at an E:T ratio of 10:1) (Fig. 6) This level of killing was comparable to that observed with FLU-stimulated T cells and FLU peptide-loaded T2 targets, performed at the same time (Fig. 6, graph "Flu-After vaccine).

Discussion: In this study, we have shown that HDI can increase both the frequency of tumor-reactive T cells initially activated by a cancer vaccine and the ability of these cells to kill tumor-antigen bearing targets.

We noted that the number of tumor-reactive T cells measured by tetramers was often higher than found using the ELISPOT assays (compare Fig. 1, 3, and 5). Such discrepancies have been noted before and may be due to T cells that are anergic or senescent or make TH2/TC2 cytokines, rather than IFN-γ, in response to peptide stimulation. [29,30,33] We have also found that a significant number of peptide-reactive T cells undergo activation-induced cell death[34] upon re-stimulation by peptides in the ELISPOT plate (unpublished results) and this phenomenon could also partially account for the lower numbers of antigen-specific cells found in the ELISPOT assays.

IFN-α is one of the oldest cytokines that has been characterized and used for immunotherapeutic purposes.[36] It has pleiotropic effects on immune responses. However, it is unclear how HDI is acting to so strikingly affect the vaccine-induced immune responses. IFN-α increases the level of MHC expression on both melanoma cells and professional APCs such as dendritic cells (DCs).[36] Consequently, residual melanoma cells in the patient may become able to directly activate g100-reactive T cells previously activated by the vaccine. Alternatively these T cells may be reactivated by DCs that indirectly present gp100 antigens that have been shed by residual melanoma cells. IFN-α is also known to prevent activation-induced cell death of T cells.[37] If gp100-reactive T cells are being chronically activated by gp100 antigens *in vivo*, the numbers of these cells may be limited by ongoing apoptosis. Since the number of antigen-specific T cells represents the difference between the number that are proliferating and the number

that are dying,[34] apoptotic blockade would lead to increased numbers of tumor-reactive T cells. Tough[38] showed that IFN-α causes bystander proliferation of $CD8^+$ T cells, which may be another mechanism whereby gp100-reactive T cells reappear in the blood after HDI. This effect has recently been shown to be mediated indirectly through IL-15 possibly released by dendritic and stromal cells in response to IFN-α,[39] which is consistent with our inability to mimic the results by directly adding IFN-α to T cell cultures.

The more potent responses seen in the *in vitro* CTL assays were mirrored in the clinical responses of the patients. M335 especially had suffered disease progression after IFN-α alone, and during vacination, but had a remarkable clinical response when HDI was administered after vaccination (Fig. 4). The mechanism by which the anti-tumor responses were made more potent by HDI is unclear. Although IFN- α is known to activate the lytic machinery and make T cells more potent CTLs,[40] increased CTL activity in our experiments was noted 8 days after the cells had been removed from the patient and cultured in the absence of IFN- α. The effect is due to an *in vivo* process and we have not been able to turn non-cytotoxic gp100-reactive T cells induced by vaccines into potent CTLs by the addition of IFN-α to *in vitro* cultures (data not shown).

The change in the quality of the anti-gp100 T cell response after HDI is reminiscent of observations we have recently reported in mice.[41] In a study of *in vivo* superantigen (SAG) responses, we found that weakly SAG-reactive T cells were stimulated just as well as strongly-reactive T cells *in vivo*, when inflammatory cytokines such as TNF-α or IFN-γ were blocked. Otherwise, strongly-reactive T cells dominated the SAG responses, as would be expected for a maturing immune response.[42] We

suggested that immune responses are "focused" by strongly-reactive T cells through the production of inflammatory cytokines that inhibit weakly-reactive T cells and account for the "affinity maturation" of an immune response. Applying this hypothesis to the observations reported here, we suggest that vaccines composed of tumor antigens that are also auto-antigens, such as gp100, stimulate a broad range of weakly reactive T cells (because of low antigen-binding affinity to the T cell receptor (TCR)[43]) which do not produce high levels of inflammatory cytokines or kill tumor cells *in vivo*. The addition of HDI provides an inflammatory signal that inhibits the function of these weak tumor-reactive T cells and allows the more strongly-reactive T cells to dominate and kill tumor cells. More evidence for this hypothesis will be a subject for future studies.

Our studies also provide an explanation for the well-known association of auto-immune diseases and acute infections. The endogenous production of IFN-α is part of natural host defense mechanisms against infections.[36] In the same way that potent anti-gp100 responses, which are essentially auto-immune responses[44] resulted after HDI, other auto-reactive T cells that are activated during infections may be made more potent by endogenous IFN-α and mediate clinical autoimmunity.

Our results suggest that the use of HDI after cancer vaccines composed of autoantigens is an effective strategy for improving the therapeutic efficacy of vaccines. We anticipate that this approach will be useful, not just for melanoma, but for any immunologically susceptible tumor. HDI offers a way to overcome a major problem associated with current vaccines - namely the transience of the induced responses - and to "focus" the vaccine-induced responses onto potent CTLs that can kill tumor cells. The optimal timing of HDI after vaccination, the optimal dose of IFN-α, and the number of

times the combination of vaccination and HDI should be repeated are subjects of ongoing investigation.

Acknowledgements: We thank Schering, Canada for providing high-dose Interferon-α for the 7 patients in this study.

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Table 1: Patient characteristics

Patient No.	Age/Sex	Time from last vaccination to IFN-α	Initial disease	Disease at time of IFN-α	Current Status	Peak frequency of gp100-reactive T cells during vaccination*	Peak frequency of gp100-reactive T cells during HDI
M136	52/M	8 months	Lung, LN**	NED***	NED	$1/5x10^4$	$1/1x10^5$
M302	53/F	3 months	Skin metastases	NED	NED	1/510	1/263
M246	47/F	7 months	LN	NED	NED	$1/1x10^5$	$1/1x10^5$
M237	49/M	8 months	LN	NED	NED	1/6667	1/1667
M166	33/M	6 months	Mesenteric Mass	Gluteal mass	Clinical regression	1/6270	1/1111
M335	32/F	1.5 months	LN, skin, breast	LNs, skin, lung	Clinical regression	1/588	1/351
M260	64/M	17 months	LN	Lung	Lung (no change)	$1/2x10^4$	$1/1x10^5$

* The peak frequency was the highest number of spots during at any time-point during active vaccination.
ELISPOT assays wereperformed as described in the materials and methods and the average of three replicate wells is reported as 1/(average spot number/10^5 plated cells).
**LN = lymph node ***NED=no evaluable disease

Table 2: Toxicity, treatment delays, and dose reductions in patients receiving HDI after vaccination.

	Grade 3*	Grade 2	Total
Constitutional Symptoms	1/7	3/7	4/7
Vitiligo	0/7	1/7	1/7
Elevated Liver Function Tests	1/7	4/7	5/7
Granulocytopenia/leukopenia	1/7	6/7	7/7
Neurologic Toxicity	1/7	1/7	2/7
Dose reduction			7/7
Dose Delay			7/7

*The delivery of HDI was modified for each patient on the basis of common toxicity criteria,[15] with 4 being the most severe, necessitating stopping treatment. A 33% reduction of dosage occurred after the first treatment interruption and a 66% reduction from baseline dose occurred after the second. No patients had a third treatment interruption that would also have required removal from treatment.

FIGURE LEGENDS

Figure 1: High-dose IFN-α recalls tumor-reactive T cells previously activated by vaccines. PBMC from patients M166 AND M335 were stimulated with the mixture of HLA-A*0201 binding gp100 peptides (gp100:209-2M and gp100:280-9V) for 8 days as described in the materials and methods. The cells were then stained with the respective phycoerythrin-labeled tetramers and CD8-FITC antibodies and analyzed by flow cytometry. The percentage of $CD8^+tetramer^+$ cells, representing gp100-reactive T cells, was then determined and is indicated in the box in each dot-plot. Before starting the schedule of vaccinations, very few gp100-reactive T cells were found in both patients (baseline). Both patients responded to vaccination and the peak response is shown in the dot-plot marked "on vaccine". Before commencing HDI, gp100-reactive T cell numbers had essentially returned to baseline (follow-up). Two weeks (for M166) and 3 weeks (for M335) after starting HDI, the number of gp100-reactive T cells again increased. All cultures were carried out at the same time, using blood that had been obtained at the indicated times and then cryopreserved.

Figure 2: Clinical response to HDI of patient M166. Magnetic resonance imaging (MRI) studies of a gluteal mass (arrows), presumed on clinical and radiologic grounds to be metastatic melanoma, before vaccination (a), 3 months after completing the vaccination protocol (b), and one month after completing HDI (c). The mass was somewhat smaller after completing the vaccine protocol but essentially disappeared after HDI.

Figure 3: Increased numbers of gp100-reactive T cells after HDI measured by IFN-γ ELISPOT assays in M166. Following the final vaccination, PBMC were collected monthly for 3 months (a) and then before beginning HDI, weekly while on HDI (indicated by the double arrow), and then monthly for 3 months (b,c). The samples were then cryopreserved so that they could all be analyzed at the same time. The cells were thawed and stimulated with a mix of the gp100 peptides (gp100:209-2M and gp100:280-9V) or FLU MP peptides (c), as a control to ensure that the culture conditions were adequate to reveal memory T cells if they were present. After 8 days of culture, ELISPOT assays were performed as described in the materials and methods. Cells were reactivated on ELISPOT plates with the gp100 peptides (solid black bar) or FLU peptides (gray bar). The average and standard deviation of the number of spots from 3 replicate wells are reported.

Figure 4: Clinical response to HDI of patient M335. Computerized axial tomography (CAT) scans of left axillary adenopathy (a, b, c) (arrows) and a lung nodule (d, e, f) (arrows), presumed to represent metastatic melanoma on clinical and radiologic grounds, before vaccination (a, d), one month after completing the vaccination protocol (b, e) and one month after completing HDI (c, f). Both areas of involvement progressed through the vaccine schedule but regressed considerably after HDI.

Figure 5: Increased numbers of gp100-reactive T cells after HDI measured by IFN-γ ELISPOT assays in M335. PBMC were collected monthly during active vaccination

(months -3 and -2, indicated by the double-headed arrow labeled "vaccine"), an observation period (months -1 and 0), weekly for 4 weeks on HDI (indicated by the double-headed arrow labeled "IFNα2b"), and then for one month following completion of HDI (month 2). Cryopreserved cells were thawed and stimulated with the two gp100 peptides. After 8 days of culture, the cells were harvested and reactivated on IFN-γ antibody coated ELISPOT plates with either the gp100 peptides (black bars) or the control FLU peptides (gray bars), as described in the materials and methods. The average and standard deviation of the number of spots from 3 replicate wells is reported.

Figure 6: Enhanced killing activity of gp100-reactive T cells during HDI. Cryopreserved PBMC after vaccination, during HDI, and one month after HDI from M166 (a) and after vaccination and one month post HDI from M335 were stimulated with the gp100-209-2M and gp100:280-9V peptides for 8 days. The cells were harvested and cultured with 2000 chromium labeled T2 cells that had been coated with the gp100 peptides or with a control HIV peptide in the effector:target (E:T) ratios indicated on the X-axis. Chromium release was measured 4 hours later. The average and standard deviation of the percent lysis from 4 replicate wells is shown. Specific killing of gp100 peptide-coated tumor targets is only seen when HDI had been given to the patients. Direct addition of IFN-α to the cultures did not increase gp100-specific CTL activity (not shown). In (a), the graph on the right marked "Flu-post vaccine" shows the CTL activity on FLU peptide-coated T2 cells when the same PBMC from M166 had been activated by FLU peptides and indicates that the culture conditions could support specific CTL activity if it was present.